Scientific Research and Essays Vol. 6(24), pp. 5091-5099, 23 October, 2011
Available online at http://www.academicjournals.org/SRE
DOI: 10.5897/SRE10.1121
ISSN 1992-2248 ©2011 Academic Journals

Review

Recent molecular tools for detecting transgenic events in genetically modified (GM) crop products

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Accepted 19 September, 2011

Genetically modified (GM) crops are the plant varieties carrying single or multiple transgenes in their genomes modified by recombinant DNA technology. Detection of transgenic elements associated with GM crops is an important issue for their traceability in food chain and also for the risk assessments related to environment and transgene introduction into human diet. A number of methods have been developed for screening GM crop products with the aim of increasing reliability and molecular sensitivity. This review article is focused on the published methods which are mainly based on PCR and DNA hybridizations as well as biosensors as a recently utilized technology. DNA hybridization methods including probe immobilization on solid surfaces and subsequent hybridization by target DNA are variously depended on surface types, probe labeling conditions or some modifications such as the use of peptide nucleic acids (PNA). Quantitative real-time PCR (qRT-PCR) is the most routinely used and compatible method for quantification which is a crucial issue in GMO content analyses. Finally, biosensors represent more advanced assays with high detection sensitivity provided by specific transducers sense DNA hybridization events. Progress in technical implementations related to GM crop analyses will contribute not only to environmental safety but also to guarantee global market functioning and the consumer rights to choose.

Key words: Genetically modified crops, multiplex polymerase chain reaction, biosensors.

INTRODUCTION

Recombinant DNA technology has enabled the introduction of foreign DNA sequences into the crop plants to confer new traits such as pathogen and herbicide tolerance, altered amino acid and fatty acid composition, delayed ripening and modified color. By this technology, specific genes encoding those traits can be cloned from any living organisms and transferred to plant genome without requiring the long steps of variety crossing and selections used in traditional breeding. *Agrobacterium tumefaciens*-mediated and microparticle bombardment (Biolistics) techniques are the most preferred methods for gene introductions. Up to date, nearly 60 GM crop plants have been registered and offered at the markets and this number is predicted to be two-fold by 2015 (Stein et al., 2010). Some examples of GM crops with transgenic events approved by European Union are MON40-3-2 of soybean, Mon1445 of cotton, Bt11, GA21 of maize and H7-1 sugar beet. By the increase of genetically modified organisms (GMOs) in global market, new legal regulations have been constituted regarding their production, import and risk assessments. For instance, European Union (EU) countries required labeling in foods includes more than 0.9% of GMO according to a regulation called EU 1829/2003. Japan, China and Australia have previously implemented labeling for foods originated from GM crops in the market. Regulations over genetically engineered crops are not discussed in only industrialized countries but also in developing countries such as Burkina Faso, Egypt and Bangladesh (Ramessar et al., 2008) since the enhancing trade and planting area of those worldwide. 25 countries planted GM crops in a total area of 148 million hectares in 2009 (James, 2010) and 670 approvals have received for 144 events related to 24 crop species.

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Among these crops, soybean comes first in 2008 with the 65.8 million hectares global planting area (53% of global biotech area). As a result of these developments, a total of 55 countries constitute regulatory approvals for biotech crops for import for food and feed worldwide. Turkey is one of the countries that started to test GM products in the market (Ari and Çakir, 2008) and declared GMO regulations under a biosafety law in 2010. This law has been prepared by taking consideration of Cartagena Protocol on Biosafety and covered the regulations over import of GM products as well as recombinant research materials used by scientific institutions in the country. All these developments regarding the legal frameworks, increase in biotech crop plantation and most importantly labeling issue indicate that reliable detection and quantification of transgenic events in food will be crucial in parallel to their production.

GM crop samples can be determined by either detection of foreign DNA segments (such as gene, promoter or terminator regions) or the novel protein itself produced by foreign DNA introduced by gene transfers. DNA based approaches can be classified as polymerase chain reactions (PCR) and DNA hybridization based methods including microtiter well-based hybridization assays, microaarrays and biosensors. The flow chart shown in Figure 1 describes the overall process for production of a GM crop and strategies for analysis of GM trait products (food/feed) by DNA-based methods. General strategy for GMO screening is based on the detection of target sequences by their amplification or DNA hybridization by a labeled-probe. Target sequences are commonly used genetic elements carried by plant transformation vectors. Particularly, “35S promoter” of ‘*cauliflower mosaic virus*’ and terminator region of “*nopaline synthase*” (NOS) gene *of A. tumefaciens* are mostly used sequences in plant genetic engineering. Therefore, a simple PCR for 35S/NOS may not be sufficient for testing all GMOs but will probably work for many GM crops and derivatives.

POLYMERASE CHAIN REACTION (PCR) - BASED METHODS

Polymerase chain reaction (PCR) (Mullis et al., 1987) is the most practical, low-cost and widely used technique among other methods for GMO detection. It can be applied to a diverse kind of samples such as seeds, leaf, processed food/feed etc. and provides very specific and accurate results in a short time. In a standard PCR procedure, forward and reverse primers which anneal to the opposite strands of DNA are used to amplify target region by a thermostable DNA polymerase. Many PCR primers for GMO detection were developed and published within recent years and have been used by many research laboratories worldwide. Validation of these PCR-based detections can be done by using reference materials. One of the most effective uses of PCR in transgene analysis is the multiplex-PCR. Figure 2 shows a simple demonstration of standard multiplex PCR performed on Californium, Jura, Elvis and Orkan varieties of canola (*Brassica napus* L.) cultivated in Turkey (unpublished data from Arican E.). Moreover, eight different GM maize could have been simultaneously identified by a single multiplex PCR experiment (Onishi et al., 2005). This method relies on the separation of PCR products from each other on the basis of their different electrophoretical mobilities. Recently, agarose gel electrophoresis is replaced by capillary gel electrophoresis (CGE) and high performance liquid chromotography (HPLC) for more sensitive separations of PCR products. By CGE, forward primers are fluorescently labeled with different fluorescent dyes such as 6-Carboxyfluorescein (6-FAM) and tetrachloro-6-carboxyfluorescein (TET) which allow identification of each PCR products by a sequencer device. For example, a combination of capillary gel electrophoresis with laser-induced fluorescence (CGE-LIF) provided a sensitive separation of multiplex PCR products of Bt event-176 maize (García-Cañas et al., 2004). Alternatively, CGE separations have been improved by using bare fused-silica capillaries which provided high resolution and reproducible separations of DNA fragments even very small in size ranging from 50 to 750 bp. This method has been applied in genetically modified maize and soybean with a detection limit of 0.9% (Sánchez et al., 2007). An important issue related to PCR-based detection is the specificity which can be evaluated in four categories (Holst-Jensen et al., 2003). In the first category, amplified regions are 35S promoter (CaMV), nopaline synthase terminator and/or genes encoding the resistance to antibiotics used in selections. In second category, detection is carried out by the amplification of specific-genes such as ‘bar’ (phosphinotricin acetyltransferase) and CryIA(b). In category 3, junctions between promoter and genes and in category 4, junction between gene and its integration locus are targeted for the PCR amplification.

Latter method is also called event-specific which has recently been demonstrated for a numbers of maize lines for example, DAS-59122-7, MIR604, MON88017 and MON863 (Oguchi et al., 2010). These applications represent PCR target sequences and determine the specificity level of transgenic event detection. Event-specific methods either based on PCR or microarrays, provide the highest sensitivity and a 0.5% of detection limit (Kim et al., 2010; Bai et al., 2010) while the amplification of promoter regions confirms only the presence of transgenes in sample (Holst-Jensen et al., 2003). Quantification is another criteria for GMO screening since it determines the labeling limit which is required by EU countries. This can be done by comparison of the relative ratio of copy number of a reference PCR target and genomic copies of respective

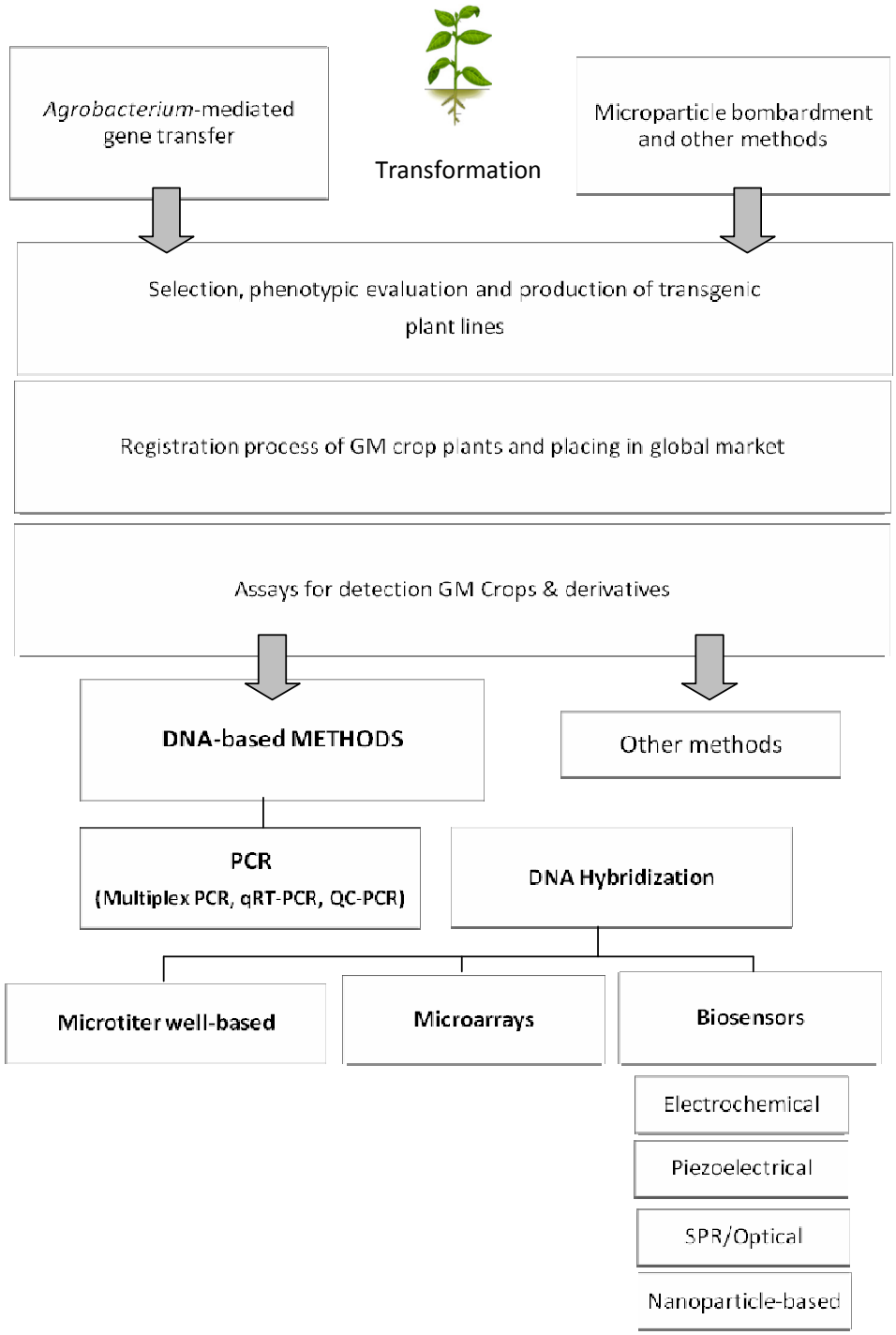

Figure 1. Process required for transgenic plant production to GM crop registration and available DNA-based strategies for analysis of these GM crops.

sequences. In the case of single insertion of introduced DNA in a diploid genome, quantification can be properly done however several copies in the genome can make the quantification unclear, if the exact numbers of copies are unknown. Soybean lectin gene is the mostly used reference in quantification experiments. Quantitative competitive PCR (QC)-PCR is one of the methods for quantification of transgenic contents in food and feeds. In this method, PCR of the target DNA and a competitor internal standard sequence is carried out in the same

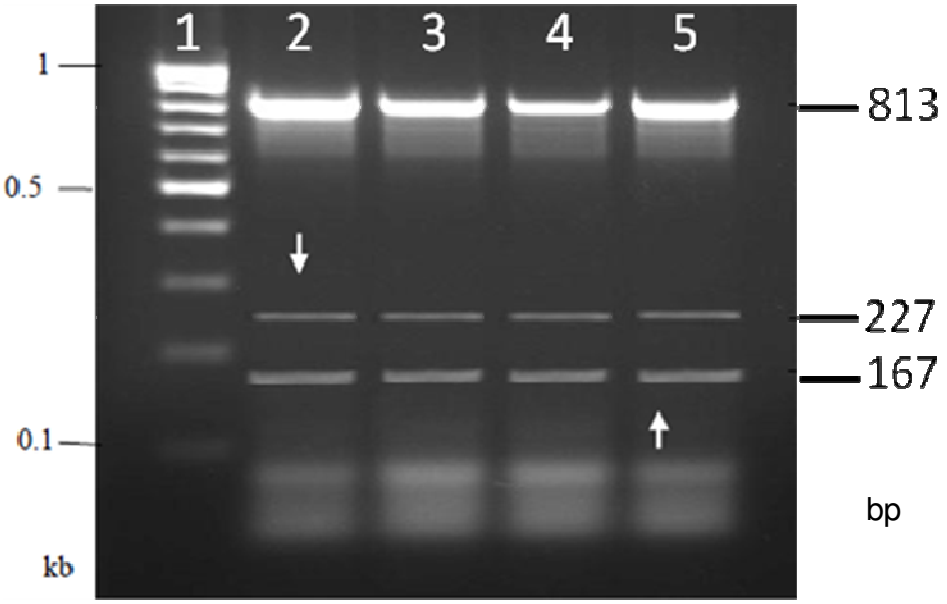

Figure 2. Multiplex PCR detection of genetically modified (GM) Canola varieties cultivated in Turkey. 35 S promoter of *Cauliflower mosaic virus* (227 bp), terminator region of *"nopaline synthase"* (NOS) (167 bp) and a housekeeping gene (Nad) (813 bp) were amplified from extracted genomic DNA from the seed material. Lanes; 1: 100 bp DNA ladder; 2: Californium, 3: Jura, 4: Elvis and 5: Orkan varieties.

reaction tube. First, products of the PCR are separated by agarose gel electrophoresis and then the quantity of target and internal standard is estimated by a regression analysis. Capillary electrophoresis can also be used here and facilitate separation period.

Real-time PCR (qRT-PCR) is the most powerful method for transgene quantification in agricultural and food products. The basis of this method is the ability to monitor the quantity of target DNA amplification in real time. This can be achieved by fluorescent dyes, - termed as *fluorochromes*-, that their binding to the DNA emits fluorescence. During the amplification, emitted fluorescence reaches a threshold level and this is correlated with the amount of target DNA in the solution, thereby enable its quantification. The way of this quantification is either based on comparison of two amplified sequences (ΔCt approach) or by a titration analysis against a standard curve. The most widely used and commercially available fluorescent dyes are Taqman ® and SYBR Green ®. During the GMO quantification; two reactions are carried out simultaneously. In one reaction, endogenous reference genes and in other reaction GM-sequences (such as CaMV and NOS) are amplified by using the same amount of template DNA. Endogenous reference genes are usually lectin gene from soybean or the invertase and zein genes from maize. Thus, transgenes quantified in the sample is measured by comparison of amplified products of reference DNA and GM-specific DNA. In each real-time PCR assay, a calibration curve is constructed for sensitive quantification by using reference materials. Mostly used reference materials with certain amounts of GM concentrations are shown in Table 1.

The main advantage of real-time PCR is that quantification data can be produced with high sensitivity without doing electrophoresis. Very little amount of target DNA in a sample can be detected by real-time PCR. These advantages and its reproducibility make the method ideal for transgene quantification in GM crop products including a variety of sample types. For instance, three different transgenic plants of maize have been shown to be distinguished by a real-time PCR assay based on SBYR green with a 1% detection limit (Marmiroli et al., 2008). Success of the quantification assays depend on the yield and quality of the DNA extracted from a sample. The samples received by a laboratory can be in a wide variety whereas the reference materials usually are in the form of powdered grains. In addition, DNA is present in low quantity in many sample types because of its loss during the food processing and refining. Temperature and pH during food processing are the major factors for DNA degradation (Gryson, 2010). Optimization of the extraction methods can be a solution to this problem (Cankar et al., 2006). Researchers pointed out the effects of components in extraction solutions and sample matrixes. Performance of the real-time PCR in GM quantification was also evaluated in respect of probe chemistries as there is an increase in

Table 1. Reference materials with known GMO contents for validation assays in quantification.

Plant	GM variety	GM concentrations (%)
Maize	Bt-176 Bt-11 Mon-810 NK603	0, 0.1, 0.5, 1, 2 and 5.
	GA21	0, 0.1, 0.5, 1, 1.7 and 4.3.
	MIR604 DAS-59122 TC1507	0, 0.1, 1 and 10.
Soybean	Roundup ready	0, 0.1, 0.5, 1, 2 and 5.
Cotton	281-24-236x 3006-210-23	1.
Rapeseed	GT73 GS40/90 MS8xRf3 Oxy235	1.
Potato	EH92-527-1	1.
Sugar beet	H7-1	0 and 100.

commercially available probe technologies (Lux™, Plexor™, Cycling Probe Technology and LNA® LNA® etc). For instance, the LNA® probes are short and appropriate when high specificity is required and where the design of a common TaqMan® probe is not possible due to sequence characteristics. Plexor™ on the other hand may be a method of choice for qualitative analysis when sensitivity and low cost were evaluated and compared using TaqMan® as a reference system (Buh et al., 2010).

DNA HYBRIDIZATION-BASED METHODS

Other methods for GM crop product detection are based on hybridization which relies on the complementary nature of two strands of DNA that hybridize each other specifically. Southern blot, microarray technology and biosensors utilize the basic principle of hybridization. In Southern blotting, isolated DNA from any sample is fixed onto a positively charged nylon membrane and probed with a double-stranded-labeled DNA which is specific to the GM-sequence. Detection is monitored by radiographically, chemiluminescence or fluoremetrically. Among these, digoxigenin and biotin-labeled DNA probes are preferred as non-radioactive systems. In microarray technology, single stranded DNA of transgene to be detected by spotting it on a solid support forming “microscobic arrays”. DNA extracted from any sample is amplified by PCR which leads to obtainment of PCR products which are later transformed to single stranded form by an exonuclease. PCR products and spotted ss-DNA are hybridized which is revealed by a fluorescent signal. By this method, several thousands of nucleic acids can be spotted on a small chip and analyzed simultaneously in a short time. Another method is proposed for the sensitive detection of 35S, NOS and lectin sequences in transgenic soybean by using bioluminometric hybridization. The method is relying on the conjugation of oligonucleotides and aequorin which is a photoprotein with a 189-amino acid long. This molecule can be detected in the presence of Ca^{+2} and represent a reporter system. For the assay, 35S, NOS and ‘lectin’ gene are amplified, biotinylated and captured by streptavidin coated microtiter wells. One strand of fixed molecules is removed to allow hybridization with oligopeptide probes specific to target sequences and a poly (dT). Subsequent binding of prepared (dA) 30-aequorin conjugates to these probes can be detected by adding Ca^{+2}. Thus, transgene content in soybean reference material has been measured by the luminescence intensity. The method is sensitive, cheaper (than real-time PCR and biosensors) and practical as it provides to test many samples in the same time by using

microtiter wells (Glynou et al., 2004).

Methods based on DNA hybridization have been mostly improved by combination with PCR to facilitate rapid detection of transgenes simultaneously. Basic mechanisms of these methods are briefly explained as follows:

Cloth-based hybridization array system (CHAS)

In this method, target GM-sequences (35S, NOS) are amplified by multiplex PCR in the presence of digoxigenin-labeled dUTP and hybridized with immobilized DNA probes onto a hydrophobic polyester cloth as a solid support. Detection was done by subsequent reactions of the anti-digoxigenin antibody-peroxidase conjugate and chromogenic substrate solution. Polyester cloth is proven to be a DNA-adsorbent surface and provided high specificity that no cross-reactions of heterologous sequences have been detected during the application. Multiplex PCR-CHAS method was tested in BT176, T14, T25, GA21 and Bt 11 of maize and roundup ready (RR) soybean (Blais et al., 2002).

Multiplex quantitative DNA array-based PCR (MQDA-PCR)

In the first part of this method, a two-step PCR procedure is carried out by using bipartite primers that carry a universal region (HEAD) equal for all different types of targets. Then, amplified fragments are hybridized with labeled probes spotted on a solid phase. The use of the bipartite primers and their universal parts is the critical point in this method and increase the uniformity of amplified PCR products (Rudi et al., 2003).

PCR-LDR (PCR-ligation detection reaction)

This method includes a primary PCR amplification followed by a solution phase LDR detection and solid phase hybridization on a universal array. Two kinds of probes are designed for the method. First probe is specific to target sequences, thus discriminative and also carries Cy3 fluorochrome at 5' termini. The second one is a common probe which is 5'-phosphorylated and contains a complementary 3'-sequence (cZipcode) that provides the hybridization on the array. Multiplex PCR products are used for LDR by using two probe types and DNA ligase in proper conditions. This step is followed by a final hybridization which is performed in the dark. Fluorescent signals on universal array can be detected by a laser scanning system. Specificity of this method is confirmed by quantifying the signal intensity for transgene and reference probes hybridized to arrays while no signal is detected for other probes (Bordoni et al., 2005).

PCR and PNA (peptide nucleic acid) arrays

PNA microarray has been developed for four types of transgenic maize (MON810, Bt11, Bt176 and GA21) and 'roundup ready soybean'. The array is built up of PNAs which have pseudopeptide chains of N-aminoethylglycine monomers allowing their hybridization with DNA. PNA probes are synthesized after their sequence determination by a peptide synthesizer, purified by HPLC and used for array preparation on commercial slides. Seven target sequences described earlier have been amplified from the samples by multiplex PCR and captured by complementary PNA probes as monitored by an Array Scanner on the basis of fluorescent intensity. The method is demonstrated that PNA array is efficient for simultaneous detections. Its sensitivity is however affected from the length of probes and their distance from the slide surface (Germini et al., 2005).

NASBA implemented microarray analysis (NAIMA)

NASBA is a method routinely used in clinical diagnosis of human pathogens and for their quantification in food and other samples. This method is proper for detection of low copy DNA and thus integrated for transgene quantification by combining with microarray technology. In the first step of the method, one tailed primer is synthesized that its 3' region is specific to target sequences and 5' region contains the promoter of SP6 RNA polymerase. Second tailed primer has the T7 RNA polymerase promoter at 5' region. These primers are used for the amplification of template sequences in multiplex PCR. By using T7 RNA polymerase, numerous copies of antisense RNA molecules are produced at the end of multiplex PCR. After several steps, -termed as NAIMA amplifications- antisense cRNA molecules are generated and fluorescently labeled for subsequent microarray analysis. Microarrays include specific complementary probes to internal segments of NAIMA products. In addition, a real-time PCR is applied to calibrate DNA copy number to assess NAIMA amplifications. The method is sensitive to provide quantitative data on the transgenic materials in a range of 0.1 to 25% (Morisset et al., 2008).

PCR-free, magnetic bead-based method with fluorescence cross-correlation spectroscopy (FCCS)

This method is developed to increase sensitivity by reducing non-target amplifications by PCR. Genomic DNAs isolated from GM soybean and tomato was digested enzymatically. A biotin-labeled probe is hybridized with the target 35S promoter and streptavidine coated magnetic beads are used to capture these target DNA in the sample. These isolated DNA targets are

hybridized with two kinds of fluorophore (rhodamine green and Cy5) labeled probes which allow the quantification of target DNA in the solution by fluorescence emissions (Zhou et al., 2009).

BIOSENSOR–BASED APPROACHES

Since the first biosensor was developed in 1960s (Updike and Hicks, 1967), those tools have become an important part of diagnostic studies. A biosensor has a “biological recognition element” associated with a physio-chemical transducer. Recently, there are a numbers of molecular recognition elements including enzymes, antibodies, nucleic acids and receptors which are being used for the development of new biosensors. For GM-detection, biosensors represent a rapid and sensitive technology and less labor-intensive than PCR-based methods. Biosensors for transgene detection can be electrochemical, optical or piezoelectric types based on their transducer component. Target DNA is captured by a complementary specific probe which is attached to the surface of this special sensor. Hybridization event thus may be translated to a value by the transducer which is associated with the probe.

Electrochemical sensors

The main feature of this biosensor is the immobilization of probe on an electrode surface such as modified cysteamine gold (Tichoniuk et al., 2008). In such a system, single stranded DNA probe was immobilized by covalent bonding with the use of carbodiimide derivatives (N-hydroxy-sulfosuccimide-NHS). In another study, electrochemical sensor was constructed on disposable screen-printed gold electrodes on which 25-mer thiol-tethered DNA probe and 6-mercapto-1-hexanol-(MHC) fixed as a monolayer (Carpini et al., 2004). Probe also carries a SH group for attachment to the electrode surface. Working principle of this biosensor relies on enzymatic assay that α-naphthyl phosphate was used as the enzymatic substrate. First, immobilized SH-probe on the electrode surface is hybridized with a biotin labeled target sequence. Subsequently, streptavidin-alkaline phosphates are added to provide putative hybrids to couple this conjugate. After several washing steps, sensors are incubated in α-naphthyl phosphate substrate solution for 20 min. Oxidation signal resulted by enzymatic product of α-naphthol which is measured by a voltmeter. Probe concentration, immobilization and hybridization time are the critical parameters of this method and optimized by researchers (Carpini et al., 2004). This type of biosensors have a sensitive detection limit (1 nmol/L), thus are very proper for testing GM crop derivatives. Electrochemical sensors are being improved by different approaches such as labeling DNA with ferrocene and thus facilitating the hybridization between captured probe and target DNA (Zhang et al., 2009).

Piezoelectric biosensors

Some materials including crystals and ceramics as well as DNA and proteins have the ability to produce electrical field in response to mechanical stress. This effect is known as “piezoelectricity” which was first demonstrated by Pierre and Jacques (1880). Piezoelectric biosensor is designed for detection of increase in the mass after a hybridization event. Quartz crystal microbalance (QSM) sensors were developed for piezoelectric sensor assays in which hybridization can be monitored in real-time (Passamano and Pighini, 2006). Immobilization of the probes onto quartz crystal surfaces were achieved by direct chemisorption of thiolated probes or attachment of biotinylated probes by streptavidine coated surfaces. Piezoelectric biosensors were tested for detection Cry1A(b) carrying maize (Passamano and Pighini, 2006) and EPSPS (5-enolpyruvylshikimate-3-phosphate synthase) transgene in roundup ready soybean (RR soybean) (Stobiecka et al., 2007). Both studies demonstrated that this kind of sensors have the ability for sensitive and specific detection of transgenic events in a sample, however their detection limit is still not at the desired level.

Surface plasmon resonance (SPR)/optical biosensors

First biosensor based on the surface plasmon resonance (SPR) transducer was commercially produced in 2002 (Mariotti et al., 2002) for specifically GMOs detection. This type of biosensors relies on optical measurement of small physical changes particularly refractive index on a metal surface. In SPR assays, this change is a result of hybridization of target DNA with the immobilized probes. Briefly, the modified refractive index is measured by the sensor and transformed to quantitative data thus allowing the monitoring hybridization without any labeling step. Similarly to other type of biosensor constructions, immobilization of the probe on gold surfaces is critical for the success of sensitive detection. Biotinylated probes have been bound to the streptavidine coated gold surface (Mariotti et al., 2002) or thiolated probes have been coupled onto crystal surfaces (Spadavecchia et al., 2005). Efficiency of SPR biosensors have been shown in diagnosis of transgenic reference materials of soybean and maize previously (Mariotti et al., 2002; Feriotto et al., 2003). Recently, an optical thin-film layer was used to monitor hybridization by reflecting altered wavelength of light upon the enzymatic catalysis (Bai et al., 2010). Six GM maize lines have been identified by this approach with high sensitivity.

Conclusion

New crops obtained by genetic engineering are getting widespread in the market all over the world. Transgenic plant cultivation and adoption of the risk assessment frameworks continue to drive the development of new analytical methods to track transgenes and their products. Most available method for identification of transgenic events in primary and secondary GM products is PCR as a rapid and inexpensive tool. Recently, biosensors with different physico-chemical transducers have been developed and experimented widely in reference GM materials. Electrochemical and piezoelectric DNA sensors are suitable for commercial use because of their sensitivity and compatibility with the other advantages as reviewed previously (Lucarelli et al., 2008). Besides, nano-particle-based and magnetic bead-based electrochemiluminescence were used to develop either disposable or reusable biosensors, respectively (Kalogianni et al., 2006; Zhu et al., 2010). Analytical performances of biosensors which are defined by detection limit, linearity range and reproducibility will be a critical issue to consider their efficiency (Zhu et al., 2010). Some of the techniques described earlier require expensive equipment and applications such as peptide synthesis, HPLC and microarray scanning. Therefore, their widespread use may not be realized in near future for many laboratories currently prefer PCR-based techniques. Besides, the quantification of foreign DNA in GM related products will be crucial for tracing genetic elements in end products.

For improving quantification, protein based methods including Western blot, ELISA and protein arrays are also in progress. In conclusion, solutions to technical limitations will facilitate efficient, standard and low-cost quantitative screening of GM crop products in near future.

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